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SP1-induced lncRNA LINC00689 overexpression contributes to osteosarcoma progression *via* the miR-655/SOX18 axis

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Abstract. – OBJECTIVE: Many findings have demonstrated long noncoding RNAs (lncRNAs) as crucial regulatory molecules in the progression of osteosarcoma. The aim of this study was to explore the roles and mechanisms of LncRNA LINC00689 (LINC00689) in osteosarcoma.

PATIENTS AND METHODS: Differential levels of LINC00689 and miR-655 in osteosarcoma samples and cell lines were analyzed by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The associations between LINC00689 expression and prognostic significance of osteosarcoma patients were analyzed using a series of statistical assays. Loss-of-function and gain-of-function assays were performed to investigate the role of LINC00689 in proliferation and metastasis *in vitro*. Bioinformatic assays, Luciferase report assays, and rescue assays were applied to illustrate the ceRNA mechanism network of LINC00689/miR-655/SOX18.

RESULTS: We found that LINC00689 expression was distinctly upregulated in osteosarcoma specimens and cell lines. MiR-655 displayed a trend of remarkably decreased expression in osteosarcoma tissues. In addition, we showed that LINC00689 could specifically interact with the promoter of SP1 and activate LINC00689 transcription. Further clinical studies indicated that higher levels of LINC00689 were associated with advanced clinical stage, positively distant metastasis, and unfavorable clinical outcome. Functional studies revealed that the knockdown of LINC00689 suppressed the proliferation, migration, and invasion of osteosarcoma cells, and promoted apoptosis. Final mechanistic investigations confirmed that upregulation of LINC00689 competitively bound to miR-655 that prevented SOX18 from miRNA-mediated degradation, thus facilitating osteosarcoma progression.

CONCLUSIONS: All our findings suggested that SP1-induced upregulation of LINC00689 promoted osteosarcoma progression by regulating miR-655/ SOX18 axis, which provided a novel insight for osteosarcoma tumorigenesis.

Key Words:
LncRNA LINC00689, MiR-655, SOX18, Osteosarcoma, Biomarker, Metastasis.

Introduction

Osteosarcoma, deriving from primordial bone-forming cells, is the most common elementary malignant bone neoplasms occurring in juveniles and young adults[1]. Distal long bones were affected by this tumor *via* the formation of neoplastic bone tissues[2]. The approximate incidence of osteosarcoma is four-five per million among the crowd. The principles of therapy of osteosarcoma have undergone impressive adjustments over the past few decades[3]. Despite of the distinct advancements in numerous therapeutic strategies, including chemotherapy, resection, and sometimes radiotherapy, the quality of life and long-term survival of patients with osteosarcoma remain impoverished, especially for these with metastasis[4-6]. In recent twenty years, although a large number of studies focused on the molecular mechanism involved in the metastasis and recrudesce of osteosarcoma, the specific mechanisms are still elusive. Therefore, further exploring the underlying mechanism is urgently required for the development of determination of cancer biomarkers and effective therapeutic strategy.

Long noncoding RNAs (lncRNAs) are new identified members of non-coding family with > 200 nt in length, and customarily considered lacking of protein coding ability[7]. Previously, they were considered to be "transcription noises". Novel studies demonstrate that some lncRNAs serve as regulators in various gene expression *via* complex epigenetic mechanisms[8]. LncRNAs have been ob-

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served in various cells experiments to be involved in numerous extensive cellular processes, including cells replication, differentiation, growth, and apoptosis[9]. Forced upregulation of some lncRNAs is found to suppress the expressions of tumor-suppressive genes to contribute to tumor formation *via* prompting growth, angiogenesis, and migration; i.e., they serve as oncogenes. Correspondingly, some other lncRNAs could promote tumor-related proteins with oncogenic activity; i.e., they function as anti-oncogenes[10-12]. Thus, the identification of more osteosarcoma metastasis-associated lncRNAs was warranted and the full elucidation of its targeting genes was also necessary.

Recently, growing biological evidence revealed a novel mechanism, the competing endogenous RNA (ceRNA) hypothesis which indicated that lncRNA transcripts could "talk" with the targeting genes of miRNAs if they have the same miRNAs binding sites[13,14]. Interestingly, lncRNA TP73-AS1 suppressed the metastasis of osteosarcoma cells *via* functioning as a sponge of miRNA-142 to increase the levels of Rac1[15]. LncRNA XIST was shown to promote osteosarcoma progression *via* modulating miRNA-21-5p/PDCD4 axis[16]. Those findings amplified the regulatory network of gene expression, providing novel clues involved in tumor progression.

LncRNA LINC00689 (LINC00689), a newly identified lncRNA, was firstly recognized as an obesity-associated gene in Northern Han Chinese[17]. Liu et al[18] firstly confirmed LINC00689 as an overexpressed lncRNA in glioma. In their functional assays, the knockdown of LINC00689 was observed to result in the suppressed ability in glioma metastasis *via* regulating miRNA-338-3p/PKM2 axis. However, its expression and possible effects in other tumors remained largely unclear. In this study, we firstly reported that LINC00689 expression was frequently upregulated in osteosarcoma. Then, we explored the clinical significance and the tumor-related function of LINC00689 *in vitro* assays. Finally, the molecular mechanism assays implied that LINC00689 works as a ceRNA by sponging miR-655.

Patients and Methods

Clinical Tissue Samples

One hundred and five paired osteosarcoma specimens and normal adjacent samples were collected during surgery at First Affiliated Hospital, Heilongjiang University of Chinese Medicine between June 2011 and November 2014 with the approvals from the Ethics Committee of our Hospital. All the patients received the written informed consents. The tissues were immediately stored at -80°C after resection.

Cells and Transfection

HOS, 143B, U2OS, MG63, Saos-2, and hFOB1.19 (as control cells) were brought from JinDun Biological corporation (Ningbo, Zhejiang, China). Roswell Park Memorial Institute-1640 (RPMI-1640) media were used to culture the cells. Moreover, fetal bovine serum (FBS; 10%) and antibiotics (1%) needed to be added into the media. For cell transfection, Lipofectamine 3000 transfection kits (Yugene, Jinan, Shandong, China) were used. The miRNA mimics or inhibitors, relevant siRNAs (including si-SP1, si-LINC00689) used in this study were all brought from Biomax Biological corporation (Kunming, Yunnan, China). The sequence of SP1, LINC00689, and RY-box transcription factor 18 (SOX18) was respectively constructed into pcDNA3.1 empty vector to overexpress corresponding gene or lncRNA by GeneYun Biological corporation (Shenzhen, Guangdong, China).

Real Time-PCR

Invitrogen TRIzol Reagents (BaofengBio, Qingdao, Shandong, China) were used to isolate RNAs. The RNAs were reverse-transcribed into cDNA with Invitrogen M-MLV reverse transcriptase (QianchengBio, Haimen, Jiangsu, China). Real Time-PCR detection for SP1, LINC00689 or SOX18 was conducted with the SYBR Green Mix kits (BaofengBio, Qingdao, Shandong, China). The SP1, LINC00689 or SOX18 expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). MiR-655 qRT-PCR detection was conducted by the use of MiRNA qPCR assay kits (JiFanBio, Daxing, Beijing, China). The miR-655 expressions were normalized to U6. RNA expressing levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers were presented in Table I.

Cell Proliferation Analyses

Cells after transfection with siRNAs (si-LINC00689) or overexpressing plasmids (ov-LINC00689) were collected and plated into plates (96-well; 1.5×10^3 cells/well). After attachment, the Dojindo Cell Counting Kit-8 (CCK-8) reagents (15 μl/well; ZhongyuanBio, Zhengzhou, Henan, China) were placed into the plates at 24, 48, 72, and 96 h. The absorbance values (OD450 nm) were detected after incubation for 1.5 h.

Table I. Sequences of the primers used for qRT-PCR.

Name	Sequence (5′→3′)
LINC00689-F	AAGAAGGTGAACGCTGGAGA
LINC00689-R	CTGGGAAGTGTAACGGCAAT
miR-655-F	CCGCGATAATACATGGTTAACCTC
miR-655-R	GCAATTGCACTGGATACGCA
SOX18-F	CCTGTCACCAACGTCTCGC
SOX18-R	CAAAGCCATAGCGCCCTGA
U6-F	GCTTCGGCAGCACATATACTAAAAT
U6-R	CGCTTCACGAATTTGCGTGTCAT
GAPDH-F	CTCCTCCTGTTCGACAGTCAGC
GAPDH-R	CCCAATACGACCAAATCCGTT

Colony Formation

Cells treated with siRNAs (si-LINC00689) or overexpressing plasmids (ov-LINC00689) were placed into plates (6-well; 8×10^2 cells/well) and cultured for 2-3 weeks. Until the colonies were seen under naked eyes, 0.1% crystal violet was utilized to treat the colonies for 25 min. Then, the colonies were washed using phosphate buffer saline (PBS) and counted by a microscope.

Detection of Apoptosis

The cell apoptosis was determined by flow cytometry using apoptosis analyses kits (BaoFeng-Bio, Wuhan, Hubei, China). About 6.5×10^5 designated siRNAs (si-LINC00689) or overexpressing plasmids (ov-LINC00689) treated-cells were resuspended in 1 × Binding Buffer (450 μl). Afterwards, 10 μl Annexin V-fluorescein isothiocyanate (FITC) and 5 μl Propidium Iodide (PI) were added into the cells, and then mixed gently for 25 min in the light-proof condition. The cells were then detected by a flow cytometer machine.

Caspase 3/9 Activity Analyses

MG63 or 143B cells after treatment were lysed with lysis buffer provided in the Abcam Caspase 3/9 activity assay kits (JiedaBio, Hangzhou, Zhejiang, China). Subsequently, the cell lysates were centrifuged (12000 g/min, 20 min, 4°C) and the supernatants were collected. Then, DEVD-pNA (5 μl; 4 mM) provided in the kits was added into the supernatants. The mixture was incubated for 1-2 h, followed by measurement with a microplate reader (absorbance at 405 nm).

Wound-Healing Assay

MG63 or 143B cells after treatment were planted into plates (24-well; 6×10^5 cells/well). On the second day, the cell confluence was near 100%. Then, single confluent cell layers were scratched by 200 μl tips. Zero and 48 h after the wounds were generated, the width of the wounds was imaged by a microscope.

Transwell Assay

Matrigel (100 μl) was firstly utilized to treat the Corning transwell inserts (8 μm pore size; Shang-JinBio, Binhai, Tianjin, China). Subsequently, the treated MG63 or 143B cells (1.5×10^5 cells) were resuspended in 200 μl serum-free media and the cell suspensions were placed into the chambers. Media plus 15% serum was placed in the bottom wells of the plates. After 36 h culture, 0.1% crystal violet was utilized to treat the invaded cells and the stained cells were counted by a microscope.

ChIP Assay

Millipore EZ ChIP kits (Dongyuan, Hefei, Anhui, China) were used for carrying out the ChIP assays. In short, formaldehyde (1%) was used for treating MG63 cells for 12 min at 27°C, followed by being treated using glycine (125 nM). Then, we added cell lysis buffer into the treated cells, and an ultrasonic apparatus was then applied to shear the chromatins into 200 to 400 bp DNA fragments. The DNAs were then precipitated by using anti-SP1 antibody (CST, Danvers, MA, USA) or treated using mouse IgG (as negative control; Bohong, Changsha, Hunan, China). The precipitated DNAs were then eluted and analyzed by qRT-PCR.

Subcellular Fractionation Assay

We used Thermo Fisher Scientific PARIS kits (Kesheng, Xiamen, Fujian, China) to isolate the nuclear and cytoplasmic fractions. Briefly, the fractionation buffer was added into MG63 cells. After incubation at 4°C for 15 min, the cells were centrifuged for 5 min at 4°C with 500 × g. The supernatants were cytoplasmic fractions, and dis-

ruption buffer was added into the pellets of nuclear fractions. Finally, RNAs were extracted from nuclear and cytoplasmic fractions, and the expression of LINC00689 was determined by qRT-PCR. U6 or GAPDH was used as an internal control of nuclear or cytoplasmic RNAs, respectively.

RNA Pull-Down Assay

The biotin-labeled LINC00689 (LINC00689-biotin) and negative control (control-biotin) were bought from Jima Biological Corporation (Suzhou, Jiangsu, China). The streptavidin-coupled Dynabeads (Invitrogen, Carlsbad, CA, USA) were used for incubation with LINC00689-biotin or control-biotin. Thereafter, the beads were added into MG63 and 143B cell lysates. Then, the RNA complexes bound to the beads were eluted, and the expression of miR-655 was determined by qRT-PCR.

Luciferase Reporter Assay

Luciferase reporter plasmids containing: wild-type B3 (B3 wt) or mutant-type B3 (B3 mut) of LINC00689 promoter; wild-type (LINC00689 WT) or (LINC00689 MUT) predicting binding site between miR-655 and LINC00689 of LINC00689 sequence; wild-type (SOX18 wt) or mutant-type (SOX18 mut) 3'-UTR of SOX18 mRNA, were constructed by GeneYun Biological Corporation (Shenzhen, Guangdong, China) using pGL3 Luciferase reporter empty vectors. The cells were allowed to grow in 48-well plates and the cell confluences reached 60-70%. Afterwards, ov-SP1 plasmids or miR-655 mimics and corresponding Luciferase reporter plasmids were co-transfected into the cells. Forty-eight hours post-transfection, the Promega Luciferase Reporter analyses kits (LongminBio, Hefei, Anhui, China) were used to detect the Luciferase activities.

Statistical Analysis

SPSS 19.0 software (IBM Corp., Armonk, NY, USA) software was applied to evaluate the statistical differences using the Student's *t*-test or ANOVA. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. Survival curves were assessed using the Kaplan-Meier method (with log-rank tests). Univariate and multivariate analyses were conducted to explore the independent risk factors for osteosarcoma. A $p < 0.05$ was considered to be statistically significant.

Results

Upregulation of LINC00689 Predicted Poor Prognosis in Osteosarcoma

To explore whether LINC00689 exhibited abnormal expression in osteosarcoma, we performed qRT-PCR in our cohort. As shown in Figure 1A, the expression of LINC00689 was distinctly upregulated in osteosarcoma specimens compared to matched normal tissues ($p < 0.01$). Besides, we also examined the levels of LINC00689 in osteosarcoma cells. When compared with human normal bone cell hFOB1.19 cells, LINC00689 levels in five osteosarcoma cells were higher (Figure 1B). Then, we further explored the clinical significance of LINC00689 in osteosarcoma patients *via* dividing 105 patients into two groups (high and low) based on the relative expression of LINC00689 in osteosarcoma specimens. As shown in Table II, the results revealed that high LINC00689 expression was associated with advanced clinical stage ($p = 0.008$) and positively distant metastasis ($p = 0.034$). In addition, we analyzed the prognostic value of LINC00689 in osteosarcoma patients

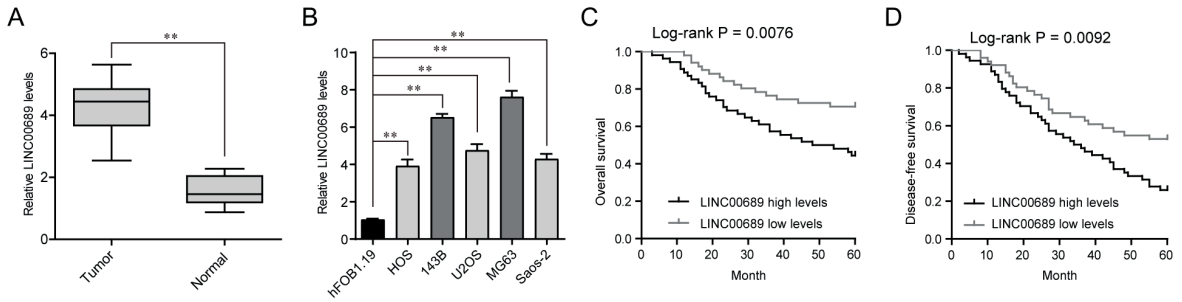

Figure 1. LINC00689 expression in osteosarcoma and its clinical significance. **A,** The expression levels of LINC00689 in 105 pairs of osteosarcoma tissues and adjacent non-tumor tissues were determined by qRT-PCR. **B,** The expression levels of LINC00689 were assessed in different cell lines by RT-PCR. **C,** and **D,** Kaplan-Meier analyses of correlations between LINC00689 expressions and overall survival (**C**) and disease-free survival (**D**) of 105 osteosarcoma patients. The median expression level was used as the cut-off. *$p < 0.05$, **$p < 0.01$.

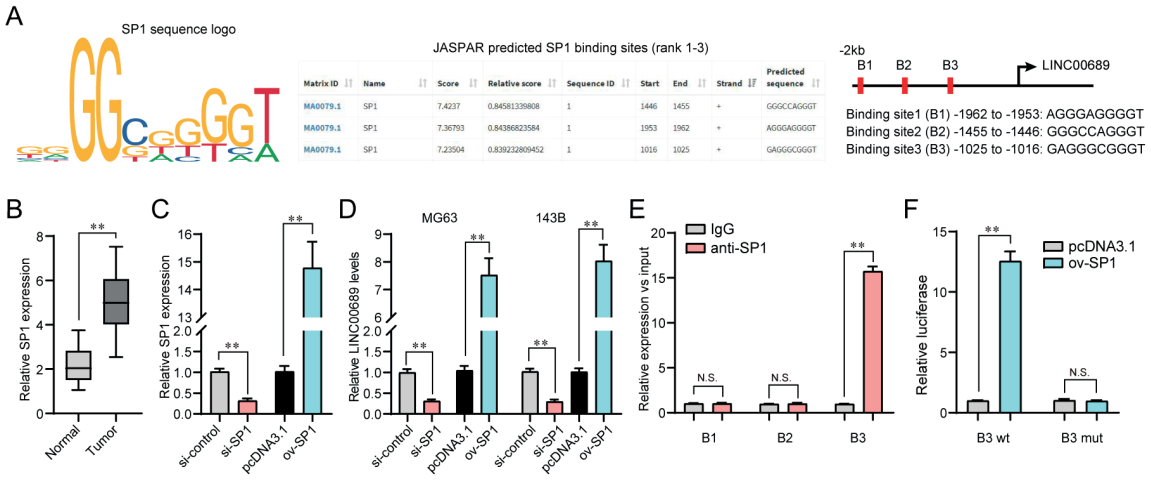

Figure 2. SP1 induced the expression of LINC00689 by promoting its transcription in osteosarcoma cells. **A,** Jaspar algorithm analyzed the binding site of SP1 on the LINC00689 promoter. **B,** qPCR detected the expression of SP1 in osteosarcoma specimens. **C,** qPCR detection. **D,** qPCR detected the expression of LINC00689 in osteosarcoma cells after various treatments. **E,** ChIP assays. **F,** Luciferase activity detection. *$p < 0.05$, **$p < 0.01$.

by the use of the Kaplan-Meier methods and log-rank tests. The results indicated that the overall survival ($p = 0.0076$, Figure 1C) and disease-free survival ($p = 0.0092$, Figure 1D) of patients with high LINC00689 expression were distinctly shorter than those with low LINC00689 expression. Subsequently, the data of univariate assays demonstrated four possible prognostic factors in osteosarcoma patients: clinical stage, distant metastasis, and high LINC00689 (all $p > 0.05$, Table III). Notably, in the multivariate assays, high LINC00689 expression was demonstrated to be an independent prognostic factor for overall survival (HR=3.103, $p = 0.009$) and disease-free survival (HR=3.446, $p = 0.005$) of osteosarcoma patients (Table III). Overall, our findings indicated

Table II. The correlation between LINC00689 and clinicopathological parameters.

Parameter	Patients, n	LINC00689 expression		p-value
		Low	High	
Age, years				0.378
<50	53	25	28	
≥50	52	29	23	
Gender				0.533
Male	69	37	32	
Female	36	17	19	
Tumor size, cm				0.217
< 8	66	37	29	
≥ 8	39	17	22	
Anatomical location				0.788
Tibia/femur	57	30	27	
Elsewhere	48	24	24	
Response to chemotherapy				0.214
Good	70	39	31	
Poor	35	15	20	
Clinical stage				0.008
IIA	67	41	26	
IIB/III	38	13	25	
Distant metastasis				0.034
Absent	74	43	31	
Present	31	11	20	

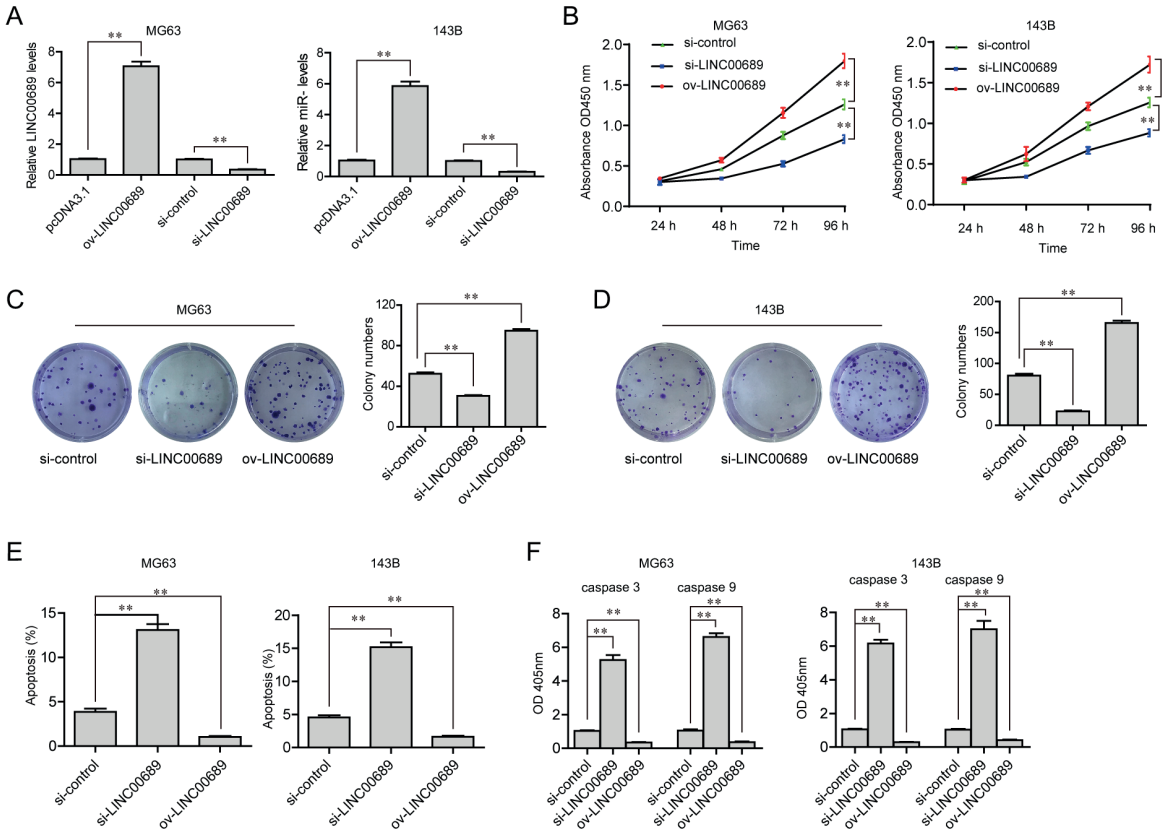

Figure 3. LINC00689 modulated the proliferation and apoptosis of osteosarcoma cells. **A,** qPCR detected the levels of LINC00689 in osteosarcoma cells after transfection with si-LINC00689 or ov-LINC00689. **B,** CCK-8 assays. **C,** and **D,** Colony formation assays (magnification: 10×). **E,** Cell apoptotic rates in MG63 and 143B cells transfected with si-LINC00689 or ov-LINC00689 were subjected to flow cytometry analyses. **F,** Caspase 3 and 9 activities were detected in osteosarcoma cells. *$p < 0.05$, **$p < 0.01$.

Table III. Univariate and multivariate Cox regression analyses for disease-free survival and overall survival in patients with osteosarcoma.

Variables	Disease-free survival			Overall survival		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Univariate analysis						
Age	1.215	0.752-1.538	0.326	1.472	0.562-1.842	0.148
Gender	1.417	0.572-2.134	0.218	1.391	0.894-1.895	0.258
Tumor size	1.379	0.833-2.018	0.137	1.446	0.954-2.231	0.117
Anatomical location	1.416	0.952-2.231	0.168	1.398	0.782-1.895	0.132
Response to chemotherapy	1.399	0.782-2.018	0.122	1.485	0.892-2.328	0.188
Clinical stage	3.218	1.327-3.872	0.006	2.894	1.138-3.432	0.011
Distant metastasis	3.462	1.482-4.102	0.003	2.958	1.218-3.687	0.009
LINC00689 expression	3.339	1.492-4.273	0.002	3.082	1.285-3.787	0.008
Multivariate analysis						
Clinical stage	3.019	1.372-3.446	0.009	3.182	1.582-3.282	0.014
Distant metastasis	2.859	1.298-3.194	0.011	2.995	1.378-3.328	0.015
LINC00689 expression	3.446	1.385-3.657	0.005	3.103	1.482-3.449	0.009

LINC00689 as an overexpressed lncRNA in osteosarcoma and a potential prognostic biomarker.

SP1 Induced LINC00689 Dysregulation by Promoting its Transcription Osteosarcoma Cells

We next attempted to explore the mechanism which contributed to LINC00689 dysregulation in osteosarcoma. Since mounting previous reports detected that transcriptional factors (TFs) might be involved in regulating lncRNAs transcription, we next applied Jaspar algorithm to analyze the possible TFs which could interact with LINC00689 promoter[19]. Among these predicting TFs, SP1, a widely-reported TF, which was able to stimulate lncRNAs aberrant expression, attracted our attentions[20,21]. The predicting binding sites (rank 1 to 3) between SP1 and LINC00689 promoter region were presented in Figure 2A. Indeed, qRT-PCR analysis demonstrated that SP1 was highly expressed in osteosarcoma tumor samples (Figure 2B). We next synthesized siRNAs targeting SP1 (si-SP1) and constructed SP1 overexpressing plasmid (ov-SP1). Then, the knockdown efficiency of si-SP1 and overexpressing efficiency of ov-SP1 were determined by qRT-PCR (Figure 2C). Afterwards, qRT-PCR assays revealed that repressing SP1 caused significantly decreased expression of LINC00689, while forced expression of SP1 contributed to remarkably increased levels of LINC00689 in osteosarcoma cells (Figure 2D). Therefore, we next carried out ChIP assays to evaluate the interaction between SP1 and LINC00689 promoter. The data validated that the complexes immune-precipitated by anti-SP1 antibodies were enriched in the predicting binding site 3 (B3) of LINC00689 promoter DNA fragments, compared with the isotype antibody control (Figure 2E). In addition, sequence containing wild-type (wt) B3 or mutant-type (mut) B3 of LINC00689 promoter was respectively constructed into pGL3 Luciferase reporter plasmids and notably increased SP1-binding activity on B3 wt reporters was observed by Luciferase reporter assays (Figure 2F). Overall, these data demonstrated that SP1 induced the transcription of LINC00689 by binding to its promoter.

Repression of Cellular Growth and Acceleration of Cell Apoptosis by Knocking Down LINC00689 in Osteosarcoma Cells

To investigate the potential functions of LINC00689 in osteosarcoma, MG63 and 143B cells were firstly treated using LINC00689 overexpressing plasmids (ov-LINC00689) or siRNAs targeting LINC00689 (si-LINC00689). The overexpressing efficiency by ov-LINC00689 and knockdown efficiency by si-LINC00689 were then identified by Real Time-PCR analyses (Figure 3A). As evidence from CCK-8 assays, the proliferation of osteosarcoma cells was remarkably promoted by ov-LINC00689, whereas LINC00689 depletion dramatically depressed the cellular proliferative rates (Figure 3B). Moreover, as the data from clonogenic assays presented in Figure 3C and 3D, inhibiting expression of LINC00689 notably repressed clonogenic capacities of osteosarcoma cells; clonogenic abilities of osteosarcoma cells were markedly enhanced by LINC00689 overexpression. Furthermore, to assess whether LINC00689 influenced the cell apoptosis, we conducted flow cytometry analyses. The data revealed that repressing the expression of LINC00689 remarkably induced cellular apoptosis, while ectopic expression of LINC00689 led to significantly decreased cell apoptotic rates (Figure 3E). Later, molecular mechanism research indicated that the activities of caspase 3/9 in osteosarcoma cells with LINC00689 deficiency were markedly higher than that of the controls, while LINC00689 overexpression notably reduced the caspases activities (Figure 3F). These results demonstrated that LINC00689 acted as an onco-promoter of osteosarcoma carcinogenesis.

Depression of Osteosarcoma Metastasis by LINC00689 Knockdown

To further study the biological functions of LINC00689 in osteosarcoma, we evaluated the impact of LINC00689 on the mobility of MG63 and 143B cells after treatment with si-LINC00689 or ov-LINC00689. As evident results from wound healing assays, the migratory abilities of the MG63 and 143B cells were notably impaired following LINC00689 silencing, while transfection with ov-LINC00689 plasmids significantly promoted the wound closures (Figure 4A). The influences on cellular invasiveness were also determined by the use of transwell assays. The data suggested that the si-LINC00689 transfection dramatically impeded the invasive capacities of osteosarcoma cells, whereas the invasion cell number was remarkably increased in osteosarcoma cells when their LINC00689 enhanced expression (Figure 4B). Therefore, these data suggest that LINC00689 functioned as a stimulator of osteosarcoma metastasis.

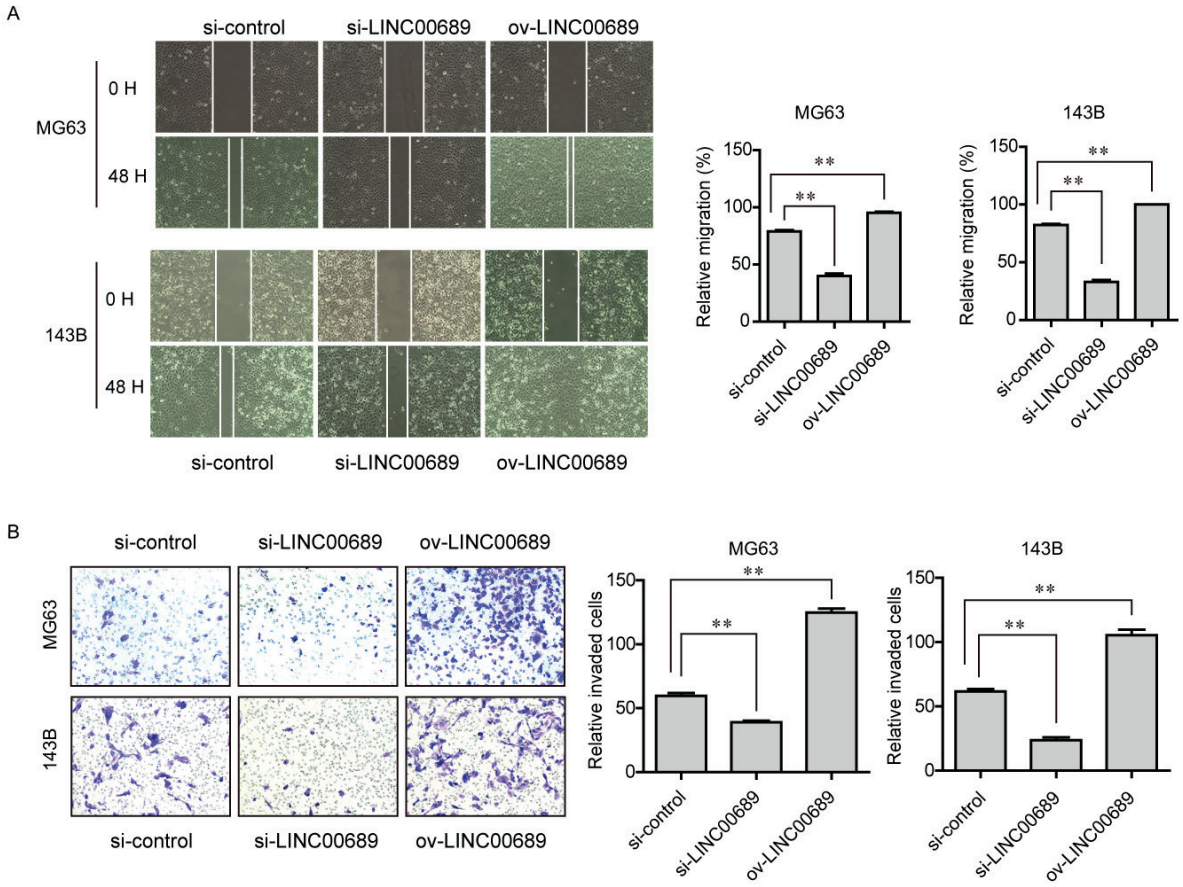

Figure 4. LINC00689 regulated osteosarcoma cell migration and invasion abilities. **A,** Wound healing assays examined cell migration (magnification: 10×). **B,** Transwell assays were conducted to assess the invasive capacities in MG63 and 143B cells transfected with si-LINC00689 or ov-LINC00689 (magnification: 40×). *$p < 0.05$, **$p < 0.01$.

LINC00689 Acted as a Sponge of MiR-655 in Osteosarcoma Cells

To reveal the molecular mechanism by which LINC00689 contributed to osteosarcoma tumorigenesis, the subcellular localization of LINC00689 was evaluated using lncLocator program. The results implied that LINC00689 was mainly located in cytoplasm (Figure 5A). Moreover, subcellular fractionation assays also revealed that LINC00689 mainly expressed in cytoplasm (Figure 5B). Hence, LINC00689 might acted as miRNA sponges to exert its functions. We thereby employed miRDB and StarBase programs to predict the potential target miRNAs of LINC00689, and the intersecting results of these two programs revealed that there were five common miRNAs (Figure 5C). Among these five miRNAs, we selected miR-655, a previously reported tumor suppressor in various cancers, for further investigation[22,23]. The predicting binding site between miR-655 and LINC00689 was shown in Figure 5D. Additionally, Real Time-PCR also confirmed that miR-655 was downregulated in osteosarcoma tumor specimens (Figure 5E). Besides, Gene Ontology (GO) and KEGG analysis using David program revealed that the target genes (predicted by miRDB program) of miR-655 were closely associated with pathways in cancer, which indicated that miR-655 was possibly relevant with malignancies regulation (Figure 5F). Therefore, we next sought to investigate whether miR-655 was able to influence the malignant behaviors of osteosarcoma cells. CCK-8 assays verified that enhancing miR-655 expression resulted in markedly inhibition of osteosarcoma cellular growth (Figure 5G). Then, wound-healing assays also showed that miR-655 overexpression contributed to the remarkable suppression of osteosarcoma cell migration (Figure 5H). Subsequently, qRT-PCR data suggested that relative LINC00689 expression in

osteosarcoma cells was suppressed by miR-655 mimics transfection, while its levels were elevated by miR-655 inhibitors transfection (Figure 5I). On the contrary, LINC00689 deficiency led to notably increased expression of miR-655, while enhancing LINC00689 expression caused remarkably decreased miR-655 expression (Figure 5J). Following Luciferase reporter assays validated that miR-655 mimics markedly reduced the Luciferase activities of osteosarcoma cells transfected with wild-type (WT) but not mutant-type (MUT) LINC00689 Luciferase reporters (Figure 5K). Furthermore, RNA pull-down assays demonstrated that LINC00689 was capable to directly interact with miR-655 in osteosarcoma cells (Figure 5L). In conclusion, these data validated that miR-655 was a direct target of LINC00689 in osteosarcoma cells.

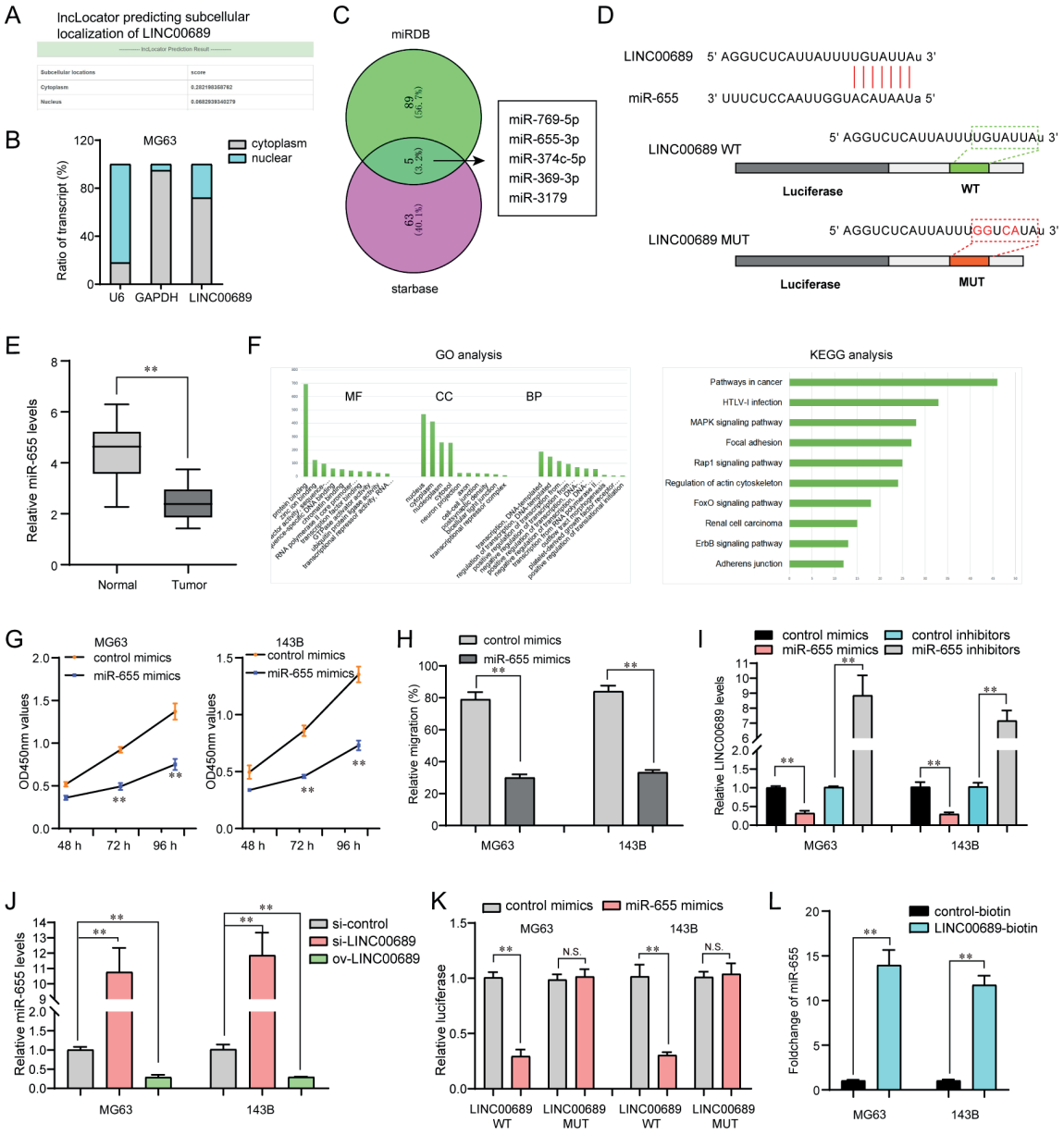

Figure 5. MiR-655 was directly targeted by LINC00689 in osteosarcoma cells. **A,** LncLocator program predicted the subcellular localization of LINC00689. **B,** Subcellular fractionation assays. **C,** The intersection of miRDB and starbase predicting results. **D,** Binding site between miR-655 and LINC00689. **E,** qPCR examined the expression of miR-655 in osteosarcoma samples. **F,** GO and KEGG analysis. **G,** CCK-8 assays. **H,** Wound-healing assays. **I,** qPCR examined the expression of LINC00689 in osteosarcoma cells after transfection with miR-655 mimics or inhibitors. **J,** qPCR measured miR-655 levels. **K,** Luciferase reporter assays. **L,** RNA pull-down. *$p < 0.05$, **$p < 0.01$.

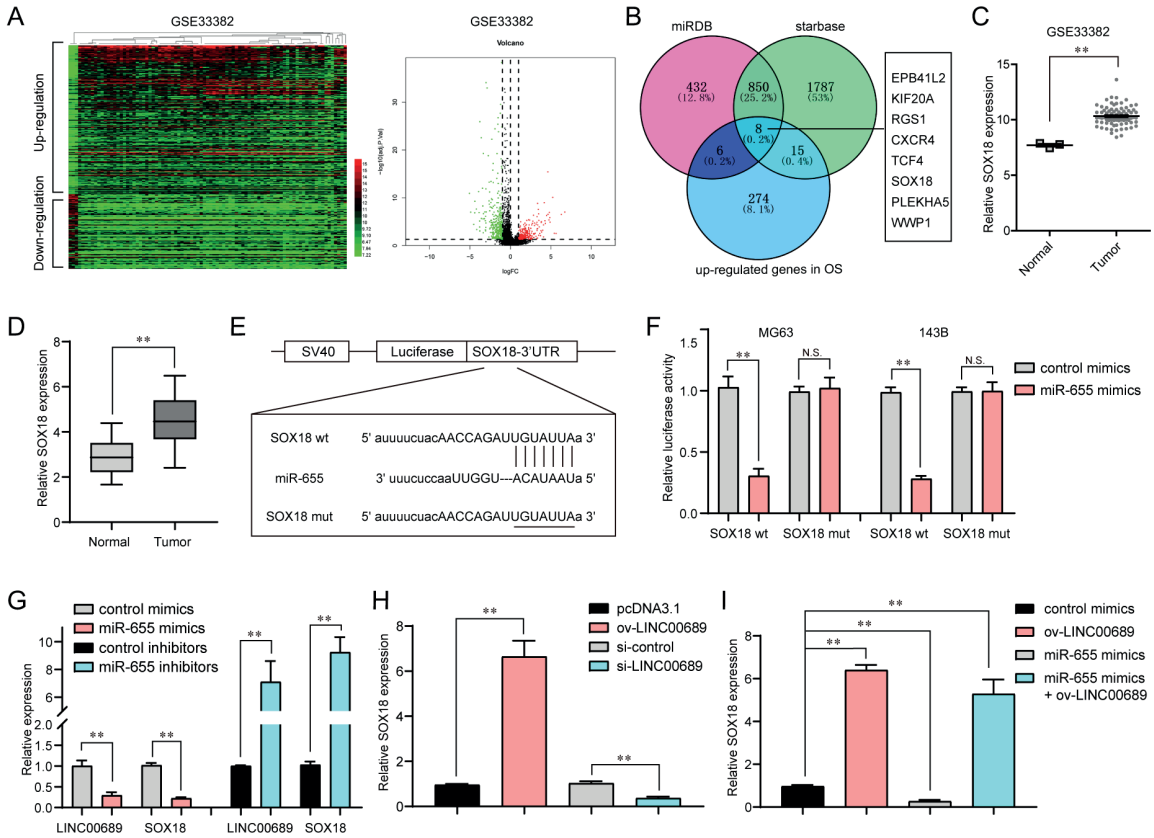

Figure 6. MiR-655 targeted SOX18 by binding to its 3'-UTR and LINC00689 regulated SOX18 via miR-655. **A,** Heatmap and volcano map of DEGs in GSE33382. **B,** The upregulated genes were intersected with the results of miRDB and starbase programs. **C,** The expression of SOX18 in GSE33382. **D,** qPCR measured SOX18 levels in osteosarcoma samples. **E,** Predicted miR-655 binding sites in the 3'-UTR of SOX18 using "starbase" algorithm. **F,** Luciferase reporter assays. **G,** qPCR examined the levels of LINC00689 and SOX18 in MG63 cells after transfection with miR-655 mimics or inhibitors. **H,** qPCR evaluated the expression of SOX18. **I,** qPCR determined the levels of SOX18 in MG63 cells after various treatments. $*p < 0.05$, $**p < 0.01$.

LINC00689 Modulated SOX18 Expression Via MiR-655 in Osteosarcoma Cells

To further elucidate the detail mechanisms by which LINC00689 regulated the malignancies of osteosarcoma, we next aimed to discover the downstream target gene of miR-655. Firstly, we conducted bioinformatics assays using GSE33382 microarray data to reveal the differentially expressed genes (DEGs) in osteosarcoma tumor samples. The heatmap and Volcano map were presented in Figure 6A. Next, the upregulated genes were intersected with the results of miRDB and StarBase programs, and there were eight common genes (Figure 6B). Among these eight genes, SOX18, an oncogene in diverse cancers, attracted our attention[24,25]. The expression of SOX18 in GSE33382 microarray data was presented in Figure 6C. qRT-PCR detection suggested that SOX18 was also upregulated in osteosarcoma tumor specimens (Figure 6D). The binding site between miR-655 and 3'UTR of SOX18 mRNA was shown in Figure 6E. To further identify whether SOX18 was able to be directly targeted by miR-655, Luciferase reporter detection was performed in osteosarcoma cells co-transfected with miR-655 mimics and SOX18 wt or mut Luciferase reporter plasmids. The data suggested that forced expression of miR-655 markedly reduced the Luciferase activities in the SOX18 wt-transfected cells, but did not change that of cells transfected with SOX18 mut, which indicated that miR-655 was able to directly interact with 3'UTR of SOX18 mRNA (Figure 6F). Besides, we performed qRT-PCR, and found that miR-655 mimics transfection could notably inhibit both LINC00689 and SOX18 expression, while miR-655 deficiency led to remarkable promotion of LINC00689 and SOX18 levels (Figure 6G). Furthermore, we also

found that enhancing expression of LINC00689 contributed to significantly increased SOX18 expression, while silencing LINC00689 caused remarkably decreased SOX18 levels (Figure 6H). Besides, the overexpression of LINC00689 notably restored the inhibition of miR-655 on SOX18 expression (Figure 6I). Taken together, our data validated that SOX18 was a target of miR-655, and LINC00689 modulated SOX18 expression *via* in osteosarcoma cells.

Discussion

The survival rates for osteosarcoma without metastasis have distinctly improved since the clinical application of multi-agent chemotherapy in the 1980s[6]. However, for the patients with metastasis, the treatment failures frequently occurred[26]. In recent years, the development of targeted therapy may change this kind of situation. However, the potential mechanism involved in tumor metastasis and progression remained largely unclear, and the sensitive biomarkers for early diagnosis and the guidance of targeted therapy were limited[27,28]. Many studies highlighted the great potentials of some lncRNAs used as therapeutic targets and cancer biomarkers due to their dysregulation and positive participation in tumor progression.

In this study, we identified a novel osteosarcoma-related lncRNA, LINC00689 which was demonstrated to be distinctly upregulated in both osteosarcoma specimens and cell lines by the use of qRT-PCR. Then, we further explored whether abnormally expressed LINC00689 had a clinical influence in osteosarcoma patients, finding that high LINC00689 expression was associated with advanced clinical stage and positively distant metastasis. Moreover, clinical survival assays revealed that patients with high LINC00689 expression exhibited a shorter five-year overall survival, suggesting LINC00689 as a positive regulator in the clinical progression of osteosarcoma. Moreover, we applied univariate and multivariate assays and confirmed that high LINC00689 expression was an independent predictor of a shorter survival in osteosarcoma patients. Overall, our findings firstly identified a novel potential diagnostic and prognostic biomarker for osteosarcoma patients.

Although evidence demonstrated the distinct dysregulation of lncRNAs in tumor samples, the potential mechanism remained largely unclear.

Various transcription factors (TFs) and several epigenetic modulators exhibited positive effects in lncRNAs dysregulation in human tumors[29]. For instance, SP1-induced upregulation of lncRNA CASC11 promoted the proliferation and metastasis of glioma cells by targeting FOXK1 by sponging miRNA-498[30]. E2F1 can activate the overexpression of lncRNA SNHG3, resulting in the improved ability in the metastatic ability of lung cancer cells[31]. Thus, we wondered whether transcription factors may be responsible for the upregulation of LINC00689. Thus, using the online TFs prediction tool JASPAR, we found that several TFs may bind to the promoter region of LINC00689, including SP1 which had three binding sites with high score. Then, we performed Luciferase reporter assays and Chip-seq assays, finding that SP1 could bind to the LINC00689 promoter region and further resulted in its transcription. Moreover, the regulator association between SP1 expression and LINC00689 expression in osteosarcoma cells was also confirmed via decreasing or increasing SP1 levels. Overall, our results revealed the possibility that SP1 upregulation in osteosarcoma was mediated by SP1.

As a newly identified lncRNA, the expression and function of LINC00689 were rarely reported in tumors. In glioma, LINC00689 was shown to be highly expressed, and its overexpression may promote the proliferation and invasion of glioma cells *via* modulating miRNA-338-3p/PKM2 axis[18]. These findings suggested LINC00689 as a positive regulator in glioma progression. In this study, we performed a series of functional assays to explore the role of LINC00689 in the cellular ability of osteosarcoma, finding that LINC00689 deletion led to distinct suppression of cell proliferation, migration and invasion, while induced apoptosis in MG63 and 143B cells. However, overexpression of LINC00689 displayed an opposite effect. Thus, our data revealed that overexpression of LINC00689 accelerated the progression of osteosarcoma, which may provide a novel therapeutic target for osteosarcoma patients.

Some reports[13] have demonstrated the possible effects of lncRNAs functioning as ceRNA for various miRNAs. This new mechanism opened up a new research field which may explain the reason causing the dysregulation of functional genes. Previously, several lncRNAs had been reported to regulate the expressions of genes by sponging miRNAs in osteosarcoma. Of note, lncRNA SNHG3, an overexpressed lncRNA in osteosarcoma, was found to promote the metastasis of osteosarcoma cells *via* absorbing miR-

151a to decrease RAB22A expressions[32]. LncRNA FBXL19-AS1 was shown to be highly expressed in osteosarcoma and promote promoted the proliferation and invasion of osteosarcoma cells by sponging miRNA-346[33]. In this study, we also wondered whether LINC00689 also act as serve as a ceRNA. The results of subcellular fractionation indicated that LINC00689 was expressed both in the cytoplasm and the nucleus, suggesting that LINC00689 may act as ceRNAs by competitively binding miRNAs. Then, the assays of two online tools revealed five possible targets of LINC00689, and our attention focused on miR-655 due to its distinct downregulation in osteosarcoma specimens and its targeting genes which were shown to strongly associated with tumor progression by the use of GO analysis and KEGG analysis. Further functional study with *in vitro* indicated that overexpression of miR-655 suppressed the proliferation and migration of MG63 and 143B cells, which was in line with the oncogenic roles in other tumors, such as lung cancer and ovarian cancer[22,34]. In addition, we performed Luciferase activity assays and pull-down system, confirming the direct binding relationships between LINC00689 and miR-655. Overall, our findings firstly confirmed that LINC00689 may display its oncogenic roles *via* sponging miR-655 in osteosarcoma cells.

Then, we further explored the potential targets of miR-655 and observed eight possible genes. In our qRT-PCR assays, we found that SOX18 expression was distinctly upregulated in osteosarcoma samples from our cohort and the samples from GSE33382. SOX18, located on 20q13.33, belongs to the family of transcription factors, which has been demonstrated to exhibit the extensive regulatory function in the embryology and the developments of lymphatic vessels[35,36]. In recent years, growing studies confirmed that many patients with various tumors displayed distinctly high levels of SOX18, suggesting that SOX18 acted as an important regulator in tumor progression. In addition, a series of functional assays also demonstrated the oncogenic roles of SOX18 in the ability of cellular growth and metastasis of several tumors, including osteosarcoma[37-39]. In this research, using Luciferase assays, SOX18 was confirmed to a direct target of miR-655. Finally, we explored the regulatory association among the above three factors, finding that upregulation of LINC00689 was able to increase the levels of SOX18, in addition, its knockdown could reduce the mRNA levels of SOX18 decreased by miR-655 mimics. Overall, our finding firstly provided evidence that LINC00689 may act as a tumor promoter in osteosarcoma at least in part by modulating miR-655/SOX18 axis.

Conclusions

Our findings firstly identified a novel osteosarcoma-related lncRNA, LINC00689 which was highly expressed in osteosarcoma and induced by SP1. Overexpression of LINC00689 promoted osteosarcoma progression through the miR-655/SOX18 axis. These observations indicated how LINC00689 induced osteosarcoma progression and may offer a potential biomarker and therapeutic target for this tumor.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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